DIRECT MICROSCOPIC AND BACTERIOLOGICAL EXAMINATION OF THE SOIL

GIACOMO ROSSI

ASSISTED BY

S. RICCARDO, G. GESUÈ, M. STANGANELLI, AND TSU KAO WANG

Royal Higher Agricultural Institute, Portici, Italy

Received for publication May 29, 1935

The object of this paper is to summarize in English the methods previously published by the writer (3–12) in Italian and German and to explain the most important results obtained with these methods.

METHODS ADOPTED IN OBSERVATIONS

Preparation of the soil sample by impression

Although the impression method is now somewhat out of favor as compared with the preparation of the soil sample by crushing, to be described later, it seems advisable to refer to it, as it is still of certain value in many cases. In putting it into practice a kind of soil impression apparatus is employed. This is essentially a device to hold a microscope slide in such a manner that it can be pressed against the surface of soil. At first the writer smeared this slide with a solution of agar or gelatin, but the present technic uses a clean slide thoroughly sterilized by being passed 50 times over a flame or, when considered necessary, also treated with distilled water.

The material remaining attached to the slide is then fixed by heat in the usual way, stained by the Conn method with carbol erythrosin (1 per cent erythrosin solution with 5 per cent phenol) employing heat, and thoroughly washed and dried.

In the case of a heavy soil, this slide is pressed against a fresh cut made with some sharp instrument. With a sandy or crumbly soil it is necessary, however, to prevent any lateral slipping. To this end metal sheets may be used with a sharp cutting edge and forcibly driven into the soil with a mallet, in this way marking out on three sides a parallelepiped of ground. Next, with another squared sheet corresponding in width to the median of the three sheets previously driven into the ground, the soil immediately above the layer which is to be sampled is lifted off, naturally after removal of the soil in front so as to ensure free space for the operation.

The soil impression may be taken parallel to the surface or obliquely or against a perpendicular face; the results do not vary greatly especially if the impression is taken at a certain depth. Impressions made at the surface are not satisfactory.

Preparation by crushing

For the crushing method a small lump of soil is crushed on the surface of a microscope slide. The crushing takes place without artificial means when the soil is sufficiently soft; otherwise, the selected fragment is placed on a glass or a porcelain slab and then sprinkled with as much distilled water as it is considered capable of absorbing; for this a few minutes will suffice. It will then be easy to obtain preparations by impression (Klatsch Preparat).

In many cases the crushing can be done between a pair of slides without using the apparatus. The drying process can be carried out before separating the two slides, and thus two prepared slides can be obtained simultaneously.

The microscopic examination in all cases is made with the objective in immersion, the preparation being covered with a slip or the oil being run directly on the preparation.

Observation of the lower face of the preparation

It is often desirable to study the lower side of a preparation as well as the upper side. In doing this a cover slip is prepared and stained by the method described hereinafter for counting cluster forms (golumerles). After the cover slip has been fixed with cedar oil on a slide with the oil between the upper face of the slide and the lower soil-free face of the cover slip, it is then examined with an objective immersed in another drop of cedar oil on the soil side of the cover slip. An easily recognizable field is then found and carefully studied, after which the cover slip is detached from the slide and replaced with the other side up, *rotating about one of its sides which is parallel with the longitudinal side of the slide.* One then has to locate the same field examined with the other side up. This is sometimes difficult but can usually be done with the assistance of marks made on the stained cover slip with a needle dipped in a violet dye. Especially valuable in relocating the field is a rough sketch of the field in question drawn on transparent paper which can be turned upside down to show the appearance of the desired field when it is to be studied in its second position.

Method of counting the cluster forms

The count of the groupings or accumulations reveals cluster forms far more frequently, as we shall see. To this end, from a very summary method based on the number of clusters present in 16 fields of the prepared slide, we have, with the aid of an expert mathematician,[1] developed one far more accurate which is carried out in the following way:

A number of ordinary glass cover slips are weighed and, to keep them distinct from one another, are placed on numbered squares on a sheet of paper. Each slip is then covered with small lumps or fragments of soil which are moistened to their full absorption capacity. Crushing is then accomplished

[1] Giovanni Candura.

with a slide or by means of the soil impression apparatus. After drying, the cover slips are stained in 1 per cent erythrosin dissolved in 5 per cent phenol, heated to steaming over a flame, washed in cold water, and dried over the flame. Each cover slip is then weighed again, the difference between this weight and the previous one giving the weight of the attached soil. The cover glass is then attached to a slide and examined under oil immersion. The clusters present in 20 fields are counted.

The Candura formula (5) is then applied, that is: taking P as the weight of the soil in grams, S as the area of the soil sample in square centimeters (*i.e.*, the area of the whole coverslip holding the soil sample), s as the ratio $\frac{S}{P}$ (specific area of the soil), a as the number (*viz.*, 20, as already stated) of the fields observed, N as the sum[2] comprehensive of the clusters seen in a fields, n the ratio $\frac{N}{a\ \pi r^2}$, that is the number of clusters per centimeter, we shall obtain:

$$n\ s = A$$

that is to say, A will be the number of the cluster forms per gram of soil.

In employing this method it is convenient to use two, four, or eight (usually four) series of 15 cover slips each and to apply the Candura formula to each slide. The fewer the cluster forms in the preparation, the larger the number of series will be needed. The object is to make a sufficient number of determinations so that the figure obtained upon averaging the results from half the cover slips will be approximately equal to that of the other half. Further details and notes of difficulties surmounted may be found elsewhere (12).

METHOD ADOPTED FOR OBTAINING MATERIAL

At present one method only is in use, *viz.*, *burial of the slides*, which has been employed by the writer (1, 4, 5, 7, 13) since about 1927.

Pairs of slides may be buried at a greater or less depth and extracted after a given time; they are then stained in the same way as soil samples prepared by crushing. Experiments have shown that the soil bacteria pass over the slide both on the upper and on the lower face, and not only the schizomycetes but also the hyphomycetes, streptothrices, and protozoa.

It is necessary to use pairs of slides because one of the two faces—the lower face in the case of the upper slide and the upper face of the lower slide—must be put out of action by the necessity for staining and observation. In other words, the lower face of the lower slide acts as the lower face of the upper slide.

[2] The authors take this opportunity of pointing out that on p. 238 of our article (12) the value of N is erroneously given as that of the *area occupied by 1 gram of soil* instead of, as on p. 15 of the article (5) which, however, must be corrected definitively: *the sum comprehensive of the clusters seen in a fields.*

At the moment of burial the slides are passed through the flame 50 times in order to purify them of any substance which might have chemotactic action on the soil bacteria, *unless it is preferred to smear them with substances of which it is desired to study the possible chemotactic capacity.*

In dealing with natural soil *in situ*, and if the earth is compact and can be sliced, the apparatus we have described for taking soil impressions at a depth will serve very well. Having made the cut at the required depth, the pairs of slides are placed in position and covered as far as possible with the slices of soil taken from the cut, which are pressed gently into place. The opening made in front of the cut is also filled in, if possible replacing the earth in the same order in which it was removed. Particularly in dealing with grassy turf it is advisable to keep the surface sods intact, putting them back into position over all the disturbed area. It is not advisable to water in this case.

METHODS OF REPRESENTATION AND DESCRIPTION

One of the greatest difficulties in these studies is to convey what we have seen to other students who do not wish to verify our observations by repeating them. Illustrations have been given in color in previous articles, but are not reproduced here because of the expense. For the benefit of those who are interested in studying them and who have, or can obtain, the preceding articles, the eight plates previously published are referred to here (in Roman numerals) according to the following scheme:

Plate I (9, plate I); Plate II (9, plate II); Plate III (10, plate VI, and 5, plate I); Plate IV (10, plate VII); Plate V (3); Plate VI (11); Plate VII (8); Plate VIII (13).[3]

As to the method of reproduction, we naturally had not much liberty of choice between a drawing from the microscope and microphotography. Plates I to IV are examples of the drawings; and Plate V, fig. 10, 11, 12, and Plate VII, fig. 49 *a*, *b*, *c*, 50, and 51 are examples of the microphotography. The latter have been rendered more explanatory by coloring. These drawings are not claimed to be absolutely accurate reproductions because of three sources of error: first, the fact that the draftsman is not always scientifically trained; second, that the reproduction of color with artists' paints is not always exact; and third, that their reproduction in printing by the ordinary three-color plate method is still more difficult.

In the preparation of these drawings we have preferred a magnification of 750 diameters rather than one of 2,000 diameters as employed by Winogradsky.

Morphological observations made over a period of approximately 8 years on the groupings of schizomycetes and other microorganisms visible in the soil can be summed up in the following way, and the groupings themselves may be classified morphologically as will be shown later. It must, however, be premised that by the term *colonies* we mean something which has practically nothing in common with those which we are accustomed to find in agar gelatine

[3] *Sup. Proc. Internatl. Soc. Soil Sci.*

cultures, particularly because they are very much smaller and barely reach the size limits of the deep-seated colonies therein immediately after development.

Filmy colonies. These mainly consist of elongated schizomycetes (bacteria or bacilli), varying in length between 1.5 and 4.2μ and in breadth between 0.4 and 1.00μ (Plates: I, fig. 6; II, fig. 1; III, fig. 1 and 4; V, fig. 6; VII, fig. 45, 46, 47, 48_1, 48_2, 48_3). Although for the most part microscopic, colonies are often found as large as the field of the microscope itself (eg., diameter 220μ); hence, when complete and intact, they correspond to, or approximate, the size limits of the colonies deep-seated in agar cultures.

They all resemble the bacterial *films* of varying consistency, sometimes cartilaginous, which are formed on the surface of nutritive liquids. They too can be folded back—possibly by the action of the apparatus or generally of the manipulations—and the edges may be superimposed.

If an intercellular substance exists, it does not take the *erythrosin staining.* These colonies are sometimes visible to the naked eye, as may be seen in figure 10 of Plate II, on which is clearly shown the folding back, at times more than one fold apparently being present There exist forms which may be called "filmy colonies," which are not clearly disclosed by the microscope (ultra-microscopic colonies?) (Plate I, fig. 3). And still other forms exist resembling folded scraps, as though developed within the irregular openings between the mineral particles into which they fit.

Schizomycetic masses in cluster form. This is by far the most frequent form and the one we regard as characteristic of the soil in which it is found. These are the most frequent apparent types of the schizomycetes in the soil, and we have here an instance of groupings, which by their nature can never be of a large number of individuals (essentially of coccic or coccobacteric forms), because, in spite of many thousand observations, these groupings have *never* been found to assume any considerable proportions, except in the case of the *giant clusters* of which mention is made in another report (13) (Plate VIII, fig. 2 and 3). The number of constituent forms, so far as it is possible to count them, hardly exceeds 30; only when they assume the appearance resembling the embryological *morula*, forming compact masses, the number of individuals must be some hundreds. One of the largest areas is also reproduced in Plate V, fig. 3. It is not difficult to observe aspects which may very well be interpreted as points of transition from forms with a small number of individuals, and even of isolated individuals, to the *morula* form.

Now if 30 may be considered as the number representing the average maximum of microorganisms composing these groups, which from now on we will call *clusters*, or *glomerules*, the minimum number varies greatly and may even be supposed to be two only. There are fields in which the groupings are numerous with a varying number of components, but where there are also present isolated individuals (*e.g.*, Plate III, fig. 11). In this case one hesitates to assume that it is either a question of new clusters being formed, as it were, from parent clusters, or one merely of remains of larger clusters broken by the soil impression apparatus or by heat or by washing. It is difficult to admit that we have here merely remains of larger colonies, since as already stated *no colonies so large have ever been found.*

But in support of the view that the clusters have a morphological individuality formed only of few individuals as mentioned, there is the fact that in the large majority of cases, the *cluster* is not independent, as if it were superficially attached to a mineral fragment, but it is at the center of something which encloses it, as shown also in the figures of Winogradsky (14). The cluster, in fact, seems as though enmeshed in a membrane of plastic material which has been crushed under the action of the apparatus, opening the "envelope" and revealing the schizomycetic grouping, as shown in figures 6, 7, 8, 9, 10 of Plate III, less well marked on figures 1 and 2 of Plate V, and very well marked on figures 30 to 37 of Plate VII. In these last the appearance is exactly that of the schizomycetic cluster escaped from an envelope broken under the pressure of the apparatus. But even where there is no gap between schiz-

omycetes and the enclosing substance (and it is impossible to tell whether this space which appears to us to be void is really filled with material, which in contradistinction to the casing is *not stained*), it is clearly seen in figures 1 to 42 of Plate VII that all the clusters are immersed in a soft, and one may say, plastic substance. This substance appears to be differently stained from the schizomycetes, with that intensity of contrast which we are accustomed to note, *e.g.*, in the twofold staining of the acidophils or in histo-cytological stainings, and according to a chromatic scale which excludes only the colors of the most highly refracting part of the spectrum.

In counting the clusters of a field under examination, we have always conformed to the rule of neglecting the isolated forms, or those linked in twos or threes only, or even more, when in the same field there were also present more numerous groupings, or when the groupings reduced to a scanty number of individuals were assembled in "nests" not differing from those composed of numerous individuals.

As regards the form, there can be no doubt that the "coccic" is the prevalent form, but the *possibility must always be kept in view* that we may have, instead, cocco-bacteria, which are frequently found in conjunction with pseudo-cocci. A very variable characteristic, however, is the size even in the same preparation, as typically seen in Plate V, fig. 2.

There may rarely be found together (Plate III, fig. 7) cocci "nested" together, bacteria and bacilli with non-typical forms, and still more rarely (Plate III, fig. 6) a few individuals clearly of bacillar type "nested" like the cocci.

All these aspects occur if a thorough examination is made of the colored figures of Winogradsky.

Streptothrices. In contradistinction to Winogradsky, we have been able to discover these in the typical hyphic and stromatic forms (Plate II, fig. 3, Plate IV, fig. 6, and Plate V, fig. 12) and also to prove their presence by the use of the buried slides method.

Hyphomycetes. These we have found very frequently. An example is given on Plate II, fig. 3, and Plate VI, fig. 8. It frequently seemed quite easy to distinguish whether the forms under observation were dead or still living forms.

Other forms of schizomycetic colonies. These are quite rare, and we think (especially when dealing with soils that are rich in decomposing vegetable matter) that such of them as might be interpreted as special forms (*e.g.*, Plate II, fig. 2, and Plate V, fig. 9) are only filmy forms developed in special conditions, whether bacteric or bacillic, according to the distinction already made.

A particular form (which there is reason to believe is due to the same type of colonies and their components) is that made up of individuals with every probability and appearance of being spore-forming (Plate II, fig. 11, Plate III, fig. 1*b* and fig. 3, and Plate VI, fig. 2).

Very occasionally, groups have been found which appear to derive from the *remains of colonies* broken up by the soil impression apparatus, and made up of individuals suggesting the Amylobacters (Plate V, fig. 8) as not showing stain at the two extremities.

Protozoa. Protozoa in the form of cysts are found isolated, but, on the other hand, free protozoa (flagellate but also other forms) are found comparatively frequently grouped in masses which only the lack of accurate knowledge makes us hesitate to call "colonies" (Plate I, fig. 2; Plate II, fig. 5 and 12; Plate III, fig. 2; Plate V, fig. 11; Plate VI, fig. 1).

We have also, in all probability, observed casings of radiolar and foraminiferous forms in soils of undoubted marine origin.

Isolated forms. To what we have already said elsewhere on the subject, we can only add that, in soil strata other than the superficial layers, compact little "crowns" of *streptococci*, or possibly streptothrices, are often observed. Chains of *streptobacteria* are more rarely discernible.

We do not deny, however, that many other almost or entirely isolated forms may be found in the soil—in fact, they are frequent; but such observations as we feel justified in recording are the following:

1. The only formations which are numerous and are constantly present under the condi-

tions already specified and under those to which we shall later refer, are the filmy colonies and the clusters.

2. *All the others*, including the streptothrices, hyphomycetes, diatoms and protozoa, may be found sporadically and in some preparations in considerable numbers. These do not, however, form in the corresponding soil anything that can justify the view that they are an important or constant factor in determining its structure.

3. None of the isolated forms in small groups (such as the "crowns" of streptococci and thus coccic, bacteric, and bacillic forms) apart from the already described filmy and clustered colonies, take the form of *colonies* even as small as the filmy and clustered kinds.

Algae. Although we cannot deny the possibility that various unicellular algae may be found in the soil, we have identified numerous species, both on the surface and at comparatively deep levels, which undoubtedly suggest only the diatoms.

SIGNIFICANCE AND IMPORTANCE OF THE FILMY COLONIES

The observations made can be classified as follows:

1. Impressions of topsoils or of soils immediately below the layer of dead leaves, where this existed.

2. Impression made by raising the layers of moss or of lichens found either on the ground or on walls or trees, thus laying bare the layer immediately beneath.

3. Removal of plants from loose or soft soil, keeping the roots intact so that a small amount of soil is brought away with them, and an impression then taken of the larger among the small soil lumps thus obtained.

4. Excavation of the soil *in situ* and impression (or removal of small lumps) at depths of 5 cm., 10 cm., 15 cm., etc.

5. Making up of pots of soil mixtures, Vesuvian sand, and leaf mould, whether sieved or otherwise, in varying proportions. Examination after varying periods and in various conditions of preservation, particularly with regard to the water content.

By direct observation we have discovered the following facts:

1. By *impressing* superficial soil when the ground is damp and the plants fresh, we may note many bacilli all similar in type to those of the filmy colonies, and sometimes even filmy colonies themselves.

2. A similar result is given by lifting the layers of moss, hepaticas, and lichens in damp, shaded places and impressing the underlying mould.

3. The filmy colonies are in direct proportion to the quantity of organic matter in a state of decomposition in the soil under examination, to the weather, and to the temperature at which the soil mixture was prepared.

Giant filmy colonies found in pots holding a large proportion of leaf mould are reproduced in Plate VII, fig. 45, 46, 47, 48_1, 48_2, 48_3.

SIGNIFICANCE AND IMPORTANCE OF THE CLUSTERED COLONIES

Observational research

With the methods described and with all proper safe-guards, we have carried out two series of tests, one by observation and the other by experiment. The principal results obtained by simple microscopic observation and by counting the clusters are as follows:

Number and distribution of clusters in Italy. The smallest number of clus-

ters observed per gram of soil was 22,032 at Parco Gussone (Portici); the greatest, 13,416,699 at Benevente and 13,548,000 at Maccarese.

In both cultivated and natural soils the number of clusters tends to diminish with depth.

In ordinary arable strata (from 0 to 30 cm.) the numerical variations found in depth are of the same order as the numerical variations in extension.

Similarly in depth a given average of clusters generally holds good for strata of approximately 30 cm. thick.

In our experiments we have found the quantity of clusters to diminish from 10 to 20 million to a few thousand in descending from the surface to a depth of 1 meter.

Geographical distribution of the clusters. Winogradsky has already demonstrated, as the result of his research work from 1926 onward, that the microflora of the regions of the globe situated in the five continents does not vary greatly qualitatively, at least under direct microscopic examination. He did not, however, consider the matter of numbers, nor did Conn or Cholodny. It is our opinion that all previous research workers concerned themselves only with looking for *given forms* (the typical zoogloea) and must have tested soils from analogous strata, as the forms we have found and described prove that those of Winogradsky, Conn, Cholodny, et al. are not the only forms which are found and which may develop in the soil.

It may be added that in soil from the neighborhood of Hong-Kong we have found the same forms as in soils from other parts of the world and also in similar numerical series.

The soil clusters in relation to soil cultivation and the duration of cultivation. There is a remarkably clear relation between the *agricultural age of the soil* (by which is meant the number of years from which its human exploitation probably dates) and *the number of clusters.* The older the soil, the greater is the number of clusters; thus very few are found in soils the cultivable strata of which are only 28 years old, as at Parco Gussone (at Portici). For example, we find that soil (from Maccarese) which has a history of cultivation dating back some 3,000 years, gives 13,548,000 clusters per gram, whereas virgin soil (from Rive Pelate di S.Polo d'Enza) gives 412,119, and soil erupted from Vesuvius in 1906 has, to date, accumulated 40,392 clusters only.

The soil of a vegetable garden—one of those Neapolitan gardens which, with the aid of water, manuring, cleaning, and sun, gives five and six crops of superb products a year—was covered by Vesuvian ash in 1906 to a depth of about 25 cm. This ash was turned in, and as a result there was an increase in the number of clusters but so small as to be almost negligible when the numbers which are known to occur in rich soils are considered. This may indicate that the formation of clusters is a very slow process and *has no relation to the immediate productive capacity of the soil.*

Experimental research

Effects of physical agents on the cluster forms. The earlier research work consisted simply in placing the soils in which the clusters had been under examination, in a bacteriological thermostat for a longer or shorter period for the purpose of re-examination. It is then seen that a rise in temperature has an influence on the turgidity of the clusters and renders their staining brighter even in the space of only 40 hours.

Subsequently we had occasion to test two soil samples which had been left undisturbed in saucers containing a little water, renewed when evaporation seemed iminent. The saucers were covered with glass slips, but the closing was by no means hermetical; consequently, conditions remained undoubtedly aerobic all the time.

Examination of these soils after 7 years showed that under these conditions of humidity the clusters still remained, and one was observed (Plate VIII, figs. 2 and 3) which could only be defined as a giant type, so large that to sketch it two representations were necessary showing different focal levels.

This, together with apparent fragments of broken-up clusters observed suggested that the humidity had, so to speak, set up germination of at least part of the clusters, that is to say, a multiplication of individuals. Some of these individuals had subsequently been unable to carry out their normal chemical processes and had degenerated and disappeared. To investigate this point, direct experiments were carried on to note the action of water on the cluster forms under four distinct conditions of humidity as follows:

1. *Humidity at air saturation point* for which the soil samples were kept in closed containers with water on the floor of the container.
2. *Humidity at saturation point of the soil.* This result is obtained in practice by immersing the soil fragment in water, subsequently removing it, allowing it to drain, and then keeping it in a container such as the one previously described.
3. *Continuous immersion of the soil sample in water*, under the same conditions as the foregoing ones, with special precautions to prevent evaporation of the water.
4. *Natural humidity*, used as a control.

The results, which agreed fairly well in the majority of instances, showed that *humidity* in three cases gave a larger number of clusters (or a greater visibility due to the greater turgidity, according to our first observations?), but imbibition in three cases quickly reduced their number and with some delay in the fourth case, whereas the immersion process concluded by reducing very considerably the number in all cases.

Longevity and the preservation of the cluster forms in agricultural soil. It had already been known[4] for some time that the bacteria, including the non-

[4] See, *inter alia*, the enquiry of GILTNER, WARD, and LANGWORTHY, H. VIRGINIA 1916 Some factors influencing the longevity of soil micro-organisms subjected to desiccation, with special reference to the soil solution. *Jour. Agr. Res.* 5: 927–942, Abs. in *Centbl. Bakt.* (II) 49: 469 (1920). These writers attribute this phenomenon to the capacity of the soil for retaining moisture in hygroscopic form in as much as there seems to be no proportionate relation between the two phenomena. The prevailing clayey nature of the soil, on the other hand, would have an effect on the phenomenon.

sporing types, preserved their vitality for a long time in soil samples kept in the air.

For our own part, from 1930 onward, we ascertained that cluster forms readily taking erythrosin stain can be observed in soil samples of different degrees of age and preservation left on the shelves of the laboratory without any special attention.

In the tables given in the various reports, serial numbers are used to indicate the different samples which we have so far been able to observe. These have been proved to retain cluster forms even after 38 years of preservation in simple glass jars and even in open cardboard containers.

The number of the cluster forms for 50 microscopic fields varied from a minimum of 2 at 20 years of age to a maximum of 149 at 19 years of age, naturally in soils markedly different.

Action of agricultural operations on a small scale. An investigation was made of the effect of different methods of cultivation. The plots in this experiment were:

1. A plot lying fallow, which naturally acted as control;
2. A plot worked with a plough;
3. A plot worked by drilling implements (Marienburg drill);
4. A plot worked with various implements.

In other respects the plots remained alike for nearly 8 years.

The results of this experiment were all negative, none of the plots showing variations great enough to be regarded as of any significance.

Experiments with buried slides. In various contributions (3, 4, 10, 13) we have given accounts of a number of experiments carried out by the method described elsewhere in this report.

The results may be summarized as follows:

On the buried slides, in soils of all kinds, both bacterial clusters and filmy colonies may be found, especially on the lower face. Much more frequently are found the schizomycetes, both in isolation and grouped in ill-defined masses, hyphomycetes, streptothrices, and perhaps also desmobacteria and protozoa. There are also microorganisms that are difficult to classify, possibly including the mycobacteria of Krassilnikow (2a).

On smearing the slides with various solutions corresponding to fertilizing substances (such as sulfate of ammonia, asparagin, peptone, mannite, rice starch) in concentrations corresponding to such as would occur if used on the soil, no chemotactic effects were to be noted, apart from the influence of the starch, which brought about an abundant increase in the streptothrices and hyphomycetes of various forms, such as have been described in full detail in the articles already mentioned.

Certain groupings of hypho- and schizomycetes are clearly characteristic; the former seem to be lifeless and in process of destruction by the schizomycetes (Plate VIII, fig. 5).

There often appear on the slides what may be called "drop-forms" (sgocciolature). They are apparently produced by drops of moisture charged with bacteria coming in contact with either the upper or the lower surface of the slide and subsequently drying and leaving the bacteria attached to the glass.

The characteristic of these drop-forms is that they have not the least appearance of "*colonies.*" In a few very rare instances and only to a very small

extent, they suggest the filmy colonies described elsewhere, but in most cases they consist of schizomycetes of various kinds irregularly distributed, the prevalent forms being bacteria and bacilli, but also including *cocci*, which in the majority of cases are characteristically *very small indeed.* Sometimes (Plate VIII, fig. 8) a considerable doubt remains as to the category of organisms to which they should be properly assigned.

The so-called colloids and their behavior in regard to anilin staining. Winogradsky has called attention to the masses, probably of colloidal nature, which also stain with erythrosin in the slides stained to show bacteria and other microörganisms. We found such material to stain much less uniformly than the microörganisms, and in Plates II, V, and VII, we have shown it as staining red, red-yellow, yellowish, or yellow-gray. We have concluded that the shades of color seen in such material were not caused by the erythrosin but were either natural or the result of heating in the course of preparation. In support of this view we have described a certain number of slides contrasted with other unstained slides of the same soil and also of similar preparations treated under heat with a 5 per cent solution of phenol (as in the case of the Conn staining formula) but without any dye.

The colors are fairly uniform with each soil, fertilizers having no modifying effect.

Relations between the clusters and the absorbing root hairs. By various methods it was found possible to obtain fragments of roots and root hairs in the same preparation with the soil and the microörganisms therein. Such experiments showed no static or numerical relations between the clusters and the absorption apparatus of the plants, but the clusters were concentrated about the roots. The preparations made under natural or artificial conditions did not differ greatly from normal preparations in which roots, root hairs, and rhizoids had been intentionally or unintentionally introduced; they might or might not contain cluster forms like any other preparation; and, like any other preparation, they contained or did not contain filmy colonies, scattered bacteria, etc.

This was the first fact that led us to conclude that the cluster forms indicate a state of repose and not a trophic state on the part of the soil schizomycetes.

Specific identification of the flora observed under the microscope. An experiment has been carried out on the basis of which, after repetition and confirmation by other experiments, we should at least be in a position to deny to the cluster forms the character of Azotobacters.

From a seedbed containing sandy soil in which wheat grains have been placed to germinate, a seedling is uprooted, one of the branches of the radicle is deprived of the apical part, and the portion with abundant absorbent root hairs is divided into two parts.

One part is placed on an object glass, and water is added without crushing the plant material; it is left for a few minutes and then dried by means of a fan.

There is a considerable deposit of material which, observed under the microscope, contains:

Scattered *cluster forms*, some reduced and others typical and very pronounced.
Groups of *cocci* in chain form.
Small groupings or accumulations of delicate bacilli.
A very large number of tiny delicate bacilli, irregularly arranged, but observed only in a few fields. In all probability they develop from pre-existing colonies as a result of the water used in making the preparation.

The second portion is placed with all the soil attached in a test tube containing 10 cc. of distilled water, and well shaken. With the Hiltner and Störmer method (2b) and the processes described elsewhere (2), it is possible to determine the quantity of Amylo- and Azotobacters, and of ammonifying and denitrifying organisms, contained in the liquid derived from the suspension obtained.

The following results were noted:

Amylobacters.....none
Azotobacters.....none
Ammonifying organisms, not less than 1,000,000 and not more than 10,000,000
Denitrifying organisms, not less than 100,000 and not more than 1,000,000

Since the soil utilized was a good deal less than a gram (it was not praticable to weigh it because of the presence of the root and the impossibility of estimating the humidity), the positive numbers observed cannot be less than those actually existing.

This means that, after all, the flora seen under the microscope have, in all probability, no connection with living and trophic Azotobacters and Amylobacters. Since the other half of this sample had revealed under the microscope the presence of typical cluster forms, the connection of these with the Azotobacters is at least a matter of some doubt.

From such observations we have drawn the conclusion that the soil bacterial clusters may probably be considered as "cysts" functionally, analogous to those of protozoa.

This last conclusion is perhaps the most important. In so far as in a future more or less remote we might find ourselves obliged to assert that the bacteria observed by us in the soil are the only types or apparent types therein really discoverable, we now think it not illogical to suppose that in the stages of repose, a large number of the schizomycetes might tend to assume cluster-like forms, which for them would be stages of encysting, analogous to those of protozoa.

From these stages they would emerge when the soil-climate brought about modifications in the environment whereby (the cyst being, so to speak, opened) the bacteria would multiply with their characteristic rapidity, and would set up in turn that specific biochemical activity which distinguishes the species to which they belong. Although we do not claim that this is a strictly ac-

curate account, it is in any case certain that this principle of interpretation of the life history of the bacteria in the soil is borne out by the following observations:

The frequency and perhaps the universality of the cluster forms in agricultural soils;

The nearly constant form of the glomerular content, without excluding a diverse form of origin;

The relatively rare occurrence of the free bacteria in the soil;

The formation of filmy colonies wherever there is vegetable matter on the point of decomposing;

The demonstrated preservability of the soil cluster forms;

The analogy shown by the soil inhabiting cluster colonies with the colonies deep-seated in agar and gelatine cultures, an analogy which does not, however, go beyond the fact that both categories may take an almost similar form while belonging to widely differing species.

A similar interpretation leads besides to the better understanding of what is meant by a stage of equilibrium of the schizomycetic soil life, which we cannot conceive as one daily and ruthless struggle; and would tend to assimilate this life to the behavior of the protozoa, thereby unifying our concept of soil life.

Thus an explanation better than any at present available, could be given of the action, on the microbial flora, of irrigation, chemical manuring, green or stable manure, and, finally, ploughing; and a better explanation of the action of heat, cold, rain, and snow. The cluster schizomycetes would be like seeds which cultivation will cause to burst open at the proper moment.

CONCLUSIONS

The main conclusions that may be regarded as the result of all the work carried out so far by the authors in connecton with the direct examination of agricultural soils, may be summed up as follows:

The method of staining of the microorganisms in the soil with acid aniline stains (erythrosin, eosin), first discovered by Conn and employed by Winogradsky, may be used for obtaining direct images of the whole of the microflora (and also of a part of the microfauna) found in the soil.

By the use of special methods known as *soil impression and soil crushing methods* with variants, it becomes possible to identify in the soil the existence of special forms of *bacterial colonies*, nearly always microscopic in character, which we have described as *filmy and cluster-like*. The former predominate where organic matter on the point of decomposing is present; the second are present in greater abundance in proportion as the soil is "aged" from the agricultural point of view.

The method of *burial of slides* in the soil for periods of varying length has proved valuable for the study of the soil microflora.

The number of *cluster forms* is determinable by the special method of counting.

The isolated bacteria and the bacterial clusters in the ground can be stained even 38 years after the securing of the specimen and after it is completely dried up.

Humidity and temperature may, even *in vitro*, exercise specific influences on the cluster forms.

It is probable that the soil bacterial clusters may be considered as "cysts" functionally analogous to those of the protozoa.

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